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Detection of novel truncated forms of human serum amyloid A protein in human plasma

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Abstract **Serum amyloid A protein (SAA) is a human plasma protein that has been recognized as potential biomarker of multiple ailments including myocardial infarction, inflammatory disease and amyloiosis. Presented here is the application of a novel immunoassay technique, termed mass spectrometric immunoassay for the detection and identification of SAA present in human plasma. Results demonstrate the ability to readily detect known SAA isotypes, and to identify novel truncated forms of SAA, in the plasma of healthy individuals and those suffering from acute and chronic inflammation. The approach represents a rapid and sensitive means for the routine structural characterization of known SAA isotypes and the discovery of associated post-translational modifications.**

Key words: Mass spectrometry; Immunoassay; Human plasma; Serum amyloid A protein; Truncation

1. Introduction

Serum amyloid A (SAA) is an acute phase protein that has in recent years become recognized as a potential biomarker of various ailments. SAA describes a complex family of four genes found in many species including humans [1]. Three of these genes are commonly expressed in humans (SAA1, SAA2 and SAA4), producing proteins that act as apolipoproteins by chaperoning in the transport of high-density lipoprotein particles [2]. During 'acute phase response' – the body's reaction to the immediate onset of inflammatory stimulus with the purpose of counteracting the challenges of tissue injury, infection and trauma [3,4] – SAA1 and SAA2 levels in plasma have been shown to increase as much as 1000-fold from basal concentration of ~1–5 mg/l [5]. Accordingly, SAA1 and SAA2 (collectively referred to as SAA or A-SAA [6]) have been recognized as particularly useful biomarkers in the assessment of cardiac arrest or other inflammatory ailments [5,7]. Moreover, SAA has long been associated with amyloid plaque formation, which has been connected with numerous dysfunctions including Alzheimer's disease and multiple sclerosis [8–10]. These plaques are the result of an accumulation of truncated insoluble amyloid fibrils, composed of the last 50–75 N-terminal amino acids of the parent SAA protein. The appearance of these amyloid fibrils is believed to be caused by a naturally occurring catabolic process of SAA by a number of cell-associated and serum proteases [11–14]. Because of this catabolic process, it is currently believed that chronic inflammatory conditions (e.g. rheumatoid arthritis) and recurrent acute phase episodes (e.g. tuberculosis) lead to more pronounced levels of truncated SAA and increased amyloid plaque formation [15,16].

Typically, conventional immunoassay approaches, and immuno-staining techniques, are used in the quantification of plasma levels and in identifying amyloid plaques in tissues, respectively [6,7]. Regarding the quantification of SAA, immunoassays are commercially available that are able to determine a composite SAA level in plasma (i.e. all isotypes of SAA), however, they are unable to readily discriminate between specific isotypes or variants of SAA [6]. Because SAA is a polymorphic protein, with SAA1 and SAA2 having three (α, β, γ) and two allelic variants (α, β), respectively [17], this inability poses a potential problem in the study of SAA in that the expression of some allelic forms has been found to be race dependent [18]. This problem is compounded even further when the possibility of producing multiple, post-translational variants of each gene product is considered.

Modern mass spectrometry (MS) techniques are able to assist in the characterization of SAA by readily discriminating between variant forms of the protein through direct measurement of molecular mass. Indeed, Ducret et al., have used a method consisting of a 2D-gel electrophoresis preparation followed by liquid chromatography/electrospray ionization MS in the characterization of SAA from the sera of an aggressively inflamed individual [19]. This work successfully demonstrated the value of using a MS-based proteomics approach in characterizing multiple known isotypes of SAA, and more importantly, in the identification of as-yet undiscovered isotypes of the protein that would have otherwise been difficult to detect and characterize using immunoassay approaches. However, although the proteomics approach used by Ducret et al. is well suited for the general analysis of large numbers of proteins in plasma, it can be improved upon in the specific by targeting only SAA from plasma, thereby eliminating the two orthogonal separation techniques (2DGE and LC) needed for preparation prior to MS. Presented here is the development of a mass spectrometric immunoassay (MSIA) in which isotypes/variants of SAA are selectively retrieved from human plasma

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Abbreviations: SAA, serum amyloid A protein; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MSIA, mass spectrometric immunoassay

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(using pan antibodies toward all forms of SAA immobilized onto the stationary phase of an affinity pipette) and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Results are given illustrating the ability to use this MSIA approach to simultaneously detect and differentiate between SAA1α and SAA2α, to identify as-yet unknown variants of the proteins, and to screen for difference is SAA composition between individuals.

2. Materials and methods

2.1. Study subjects

Blood samples collected from six individuals of Caucasian origin, four males (ages ranging from 26 to 49), and two females (ages 32 and 48). The male subjects were healthy individuals, but both females were suffering from inflammation at the time samples were collected. Human blood was obtained from subjects recruited within Intrinsic Bioprobes, Inc. (IBI), following a procedure approved by the IBI's Institutional Review Board, and after each subject had signed an Informed Consent form.

2.2. Sample preparation

Whole blood samples (100 μl) were acquired under sterile conditions through a lancet-punctured finger using two non-heparinized (50 μl volume) microcolumns (Drummond Scientific Co., Broomall, PA, USA). Each whole blood sample was immediately combined with 200 μl HEPES buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4 (HBS)) containing 0.1% Tween-20 and 2 μl of a protease inhibitor cocktail set II (Calbiochem, San Diego, CA, USA) and centrifuged for 2 min (at 7000 rpm/2500×*g*) to pellet red blood cells. The supernatant (diluted plasma, 250 μl) was decanted from each sample and transferred to a column in a 96-well titer plate for immediate analysis.

2.3. Analysis

The plasma samples were interrogated in parallel via MSIA, a hybrid proteomics approach that combines affinity capture with MALDI-TOF MS detection [20–23]. MSIA utilizes affinity pipettor tips, which can be applied to detect low-level proteins directly from crude biological fluid by concentrating and purifying the target protein via a repetitive pipetting action of the sample through the affinity tip followed by a stringent rinse protocol.

The plasma/HBS samples were addressed in parallel using an eight-barreled pipette equipped with six affinity pipette tips derivatized with monoclonal antibodies specific towards the acute phase response forms of SAA (IBI). Sample incubation consisted of 50 cycles (aspiration and dispensing) of 150 μl of the sample through each MSIA-tip. After incubation, tips were thoroughly rinsed using HBS (10 cycles, 150 μl), doubly distilled water (five cycles, 150 μl), 20% acetoniltrile/1 M ammonium acetate (10 cycles, 150 μl) and finally with doubly distilled water (15 cycles, 150 μl). Retained proteins were eluted by drawing 4 μl of MALDI matrix solution (saturated aqueous solution of sinapic acid, in 33% (v/v) acetonitrile, 0.4% (v/v) trifluoroacetic acid) into each tip and depositing the eluates directly onto a 96-well formatted hydrophobic/hydrophilic contrasting MALDI-TOF target [22]. Samples were allowed to air dry prior to insertion of the MALDI target into the mass spectrometer. The total time required for preparation of the six samples was approximately 10 min.

3. Results

Fig. 1 shows a comparison of the direct MALDI-TOF MS analysis of human plasma and an anti-SAA MSIA analysis taken of the same plasma. Using MSIA, SAA was selectively extracted/concentrated from the plasma for subsequent direct analysis using MALDI-TOF MS. The application of anti-SAA MSIA to samples taken from six individuals resulted in the detection of several SAA protein isoforms and variants. Shown in Fig. 2A are the results of the anti-SAA MSIA analyses of the samples from the four males participating in the study. The mass spectra are dominated by the presence of intact SAA1α (MW = 11 682.7) and a naturally occurring

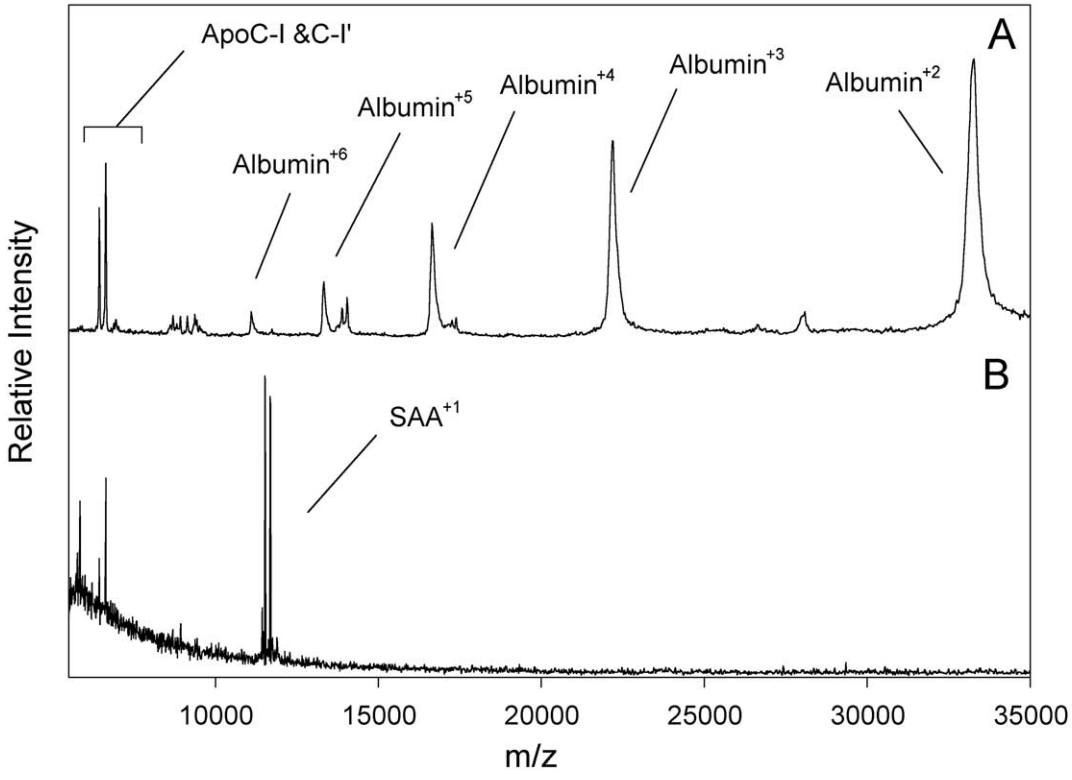

Fig. 1. A: MALDI-TOF MS of diluted human plasma. Only high-level proteins (i.e. albumin, Apo-C-I, etc.) are observed. B: Anti-SAA MSIA spectrum from human plasma.

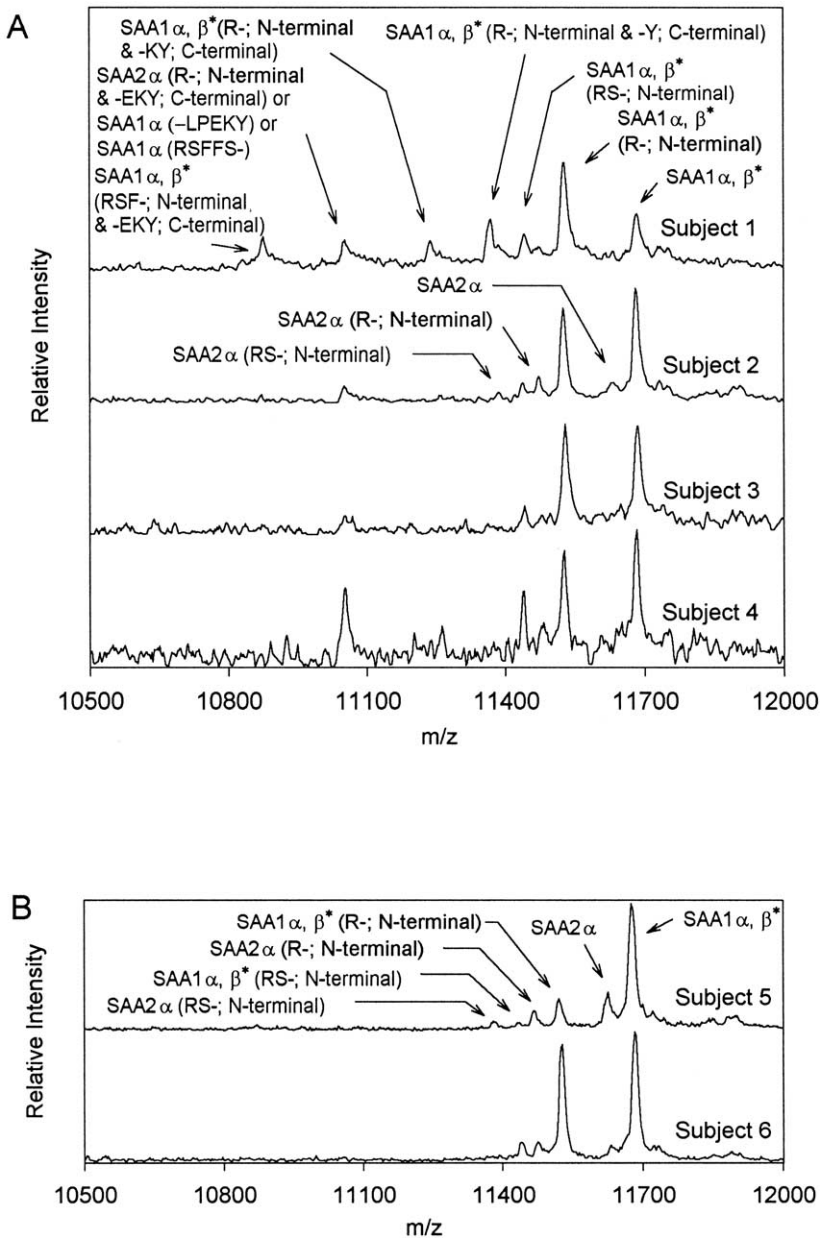

Fig. 2. A: Results of the anti-SAA analyses of the four male study participants. Intact as well as N-terminal and C-terminal truncated versions of SAA1α (MW = 11 682.7) and SAA2α (MW = 11 628.7) isotypes are present. SAA1β* (MW = 11 682.7) instead of SAA1β is also believed to be present due to an absence of signal at 11 740.7 Da. B: Results of the anti-SAA MSIA analyses of the samples from inflamed individuals. Multiple truncated peaks are still present, but are limited to the first and second N-terminal residues.

Table 1
Comparison of observed SAA species

SAA1α[a] AA1-104[b]	SAA1α* AA2-104	SAA1α* AA3-104	SAA1α* AA2-103	SAA1α* AA2-102	SAA1α* AA4-101	SAA2α AA1-104	SAA2α AA2-104	SAA2α AA3-104	SAA1α AA1-99; 6-104	SAA2α AA2-101
11 682[c]	11 526	11 441	11 363	11 235	10 871	11 628	11 472	11 385	11 052; 11 058	11 052
11 682	11 525	11 441	11 368	11 237	10 875				11 053	
11 680	11 523	11 438				11 626	11 469	11 383	11 048	
11 685	11 529	11 441							11 052	
11 684	11 529	11 443							11 054	
11 683	11 526	11 441				11 631	11 472	11 386		
11 683	11 527	11 442				11 629	11 473			

[a]SAA1α is indistinguishable in molecular weight from SAA1β*(1-104).
[b]Amino acid residues of observed SAA form.
[c]Theoretical molecular weight of observed SAA form.

post-translational modification in which the N-terminal Arg has been cleaved (SAA1α (R-; N-terminal); MW = 11 526.5). The ratios of these two dominant peaks vary from sample to sample, while several other cleavages and isoforms of SAA are also detected in varying ratios in each of the other MS traces. These protein isoforms include SAA1α (RS-; N-terminal (MW = 11 439.6)), SAA1α (R-; N-terminal and -Y; C-terminal (MW = 11 363.6)), SAA1α (R-; N-terminal and -KY; C-terminal (MW = 11 235.6)), SAA1α (RSF-; N-terminal and -EKY; C-terminal (MW = 10 872.6)), SAA2α (MW = 11 628.7), SAA2α (R-; N-terminal (MW = 11 472.6), SAA2α (RS-; N-terminal (MW = 11 385.6) and SAA2α (R-; N-terminal and -EKY; C-terminal (MW = 11 052.6)).

The results of the anti-SAA MSIA interrogation of sample from two females suffering from inflammation are shown in Fig. 2B. Subject 5 was 48-h post-surgery at the time of sample collection while Subject 6 suffers from inflammation in the form of highly aggressive chronic rheumatoid arthritis. Qualitatively, these results are very similar to those from the four male samples, but lacked the extended N-terminal or any C-terminal truncations previously seen. Both traces contained varying amounts of the intact SAA1α and SAA2α, along with multiple truncations of each. These truncations included the SAA1α (R-; N-terminal; MW = 11 526.5), SAA1α (RS-; N-terminal (MW = 11 439.6)), SAA2α (R-; N-terminal (MW = 11 472.6)) and SAA2α (RS-; N-terminal (MW = 11 385.6)). The qualitative results of all six analyses are summarized in Table 1.

4. Discussion

SAA is a polymorphic protein having several different possible alleles for each isotype, with the expression ratio of these allelic forms being race dependent. For instance, SAA1α is 80–90% dominant with SAA1γ being rarely seen in caucasians, while SAA1α, β and γ are roughly evenly dispersed in Japanese populations [18]. The spectra resulting from all of the anti-SAA MSIA were dominated by the SAA1α phenotype with no detectable signal from the SAA1γ allelic form, which is consistent with the subjects participating in this study all being of Caucasian ancestry. With regard to the presence of SAA1β, controversy still remains as to what is the correct primary sequence. The accepted literature amino acid sequence of SAA1β has an Asp residue at position 72 (MW = 11 740.7), but a conflicting sequence has been reported by Betts et al., in which a Gly is at position 72 (SAA1β*), thus changing the molecular mass to 11 682.7 Da [24] (the same molecular mass as SAA1α). The lack of ion signal at MW = 11 740.7 Da in any analysis strongly suggest that the sequence for SAA1β may be that of the conflicting sequence, SAA1β*. Given the lack of observable signal for SAA1β, and the fact that SAA1β* and SAA1α are of the same molecular mass (and share sequence homology at both termini), both of the latter species could contribute to the composite profiles in exactly the same manner and are thus labeled in Fig. 2A,B as both SAA1α and SAA1β*.

From the results of the anti-SAA MSIA analyses, we were able to detect multiple truncations of the SAA protein in each individual. The catabolism of SAA via cell-associated and serum proteases have long been known [13]. As demonstrated, anti-SAA MSIA is readily able to detect these truncated products in a manner entailing only a few experimental steps and that is relatively easy to perform. To the best of our knowledge, SAA1α, β* (RS-; N-terminal), SAA1α, β* (R-; N-terminal and -Y; C-terminal), SAA1α, β* (R-; N-terminal and -KY; C-terminal), SAA1α, β* (RSF-; N-terminal and -EKY; C-terminal), SAA2α (RS-; N-terminal) and SAA2α (R-; N-terminal and -EKY; C-terminal) detected in these analyses have not been previously reported. Regarding the individuals suffering from inflammation, the results indicate less observable truncation than in the non-inflamed individuals, which is most easily explained by an increase in the expression of the intact protein. However, the SAA profile from the individual suffering from acute inflammation (subject 5) differs from that observed from the individual suffering from chronic inflammation (subject 6) in the relative amount of SAA1α, β* and SAA1α, β* (R-; N-terminal). This phenomenon was originally observed by Raynes et al., from which it was determined that SAA without a N-terminal Arg reaches a maximum 24 h later than the full sequence isoforms [25]. Differences are also observed in the truncated patterns of the non-inflamed individuals, most prominently in profile obtained from individual 1, whom although not in any state of aggressive inflammation exhibited extensive breakdown products (relative to the other individuals). Taken collectively, these results suggest that breakdown pathways leading to the truncated products may be influenced by individual phenotype as well as state of inflammation.

5. Conclusion

In summary, anti-SAA MSIA analysis was used to identify multiple forms of SAA present in the plasma of individuals. These different forms included the known SAA1α, SAA1β and SAA2α isotypes along with various N- and C-terminal truncations of each, most of which have not been reported previously. The results obtained in this small preliminary study illustrate the need for a more concerted investigation, involving time-course studies of large numbers of individuals in order to monitor the progression and the formation of the truncated forms of SAA under inflammatory and non-inflammatory conditions and to decipher individual phenotypic contributions. Such studies may assist in the further understanding of the role of SAA with regard to inflammatory response and the problems associated with amyloidosis.

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